REVIEW

Programming for T-lymphocyte fates: modularity and mechanisms

Ellen V. Rothenberg

Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, California 91125, USA

T-cell development in mammals is a model for lineage choice and differentiation from multipotent stem cells. Although T-cell fate choice is promoted by signaling in the thymus through one dominant pathway, the Notch pathway, it entails a complex set of gene regulatory network and chromatin state changes even before the cells begin to express their signature feature, the clonal-specific T-cell receptors (TCRs) for antigen. This review distinguishes three developmental modules for T-cell development, which correspond to cell type specification, TCR expression and selection, and the assignment of cells to different effector types. The first is based on transcriptional regulatory network events, the second is dominated by somatic gene rearrangement and mutation and cell selection, and the third corresponds to establishing a poised state of latent regulator priming through an unknown mechanism. Interestingly, in different lineages, the third module can be deployed at variable times relative to the completion of the first two modules. This review focuses on the gene regulatory network and chromatin-based kinetic constraints that determine activities of transcription factors TCF1, GATA3, PU.1, Bcl11b, Runx1, and E proteins in the primary establishment of T-cell identity.

Supplemental material is available for this article.

T lymphocytes in vertebrates (T cells) develop in an almost continuous flux from hematopoietic stem and progenitor cells from fetal (or larval) life to well into reproductive maturity. Cousins of all other blood cell types, they are distinctive in the way that they maintain features that seem stem-like even as mature cells. Mature T cells retain the ability to proliferate extensively upon contact with appropriate signals and to generate clonal descendants that can persist for a large fraction of the life of the organism even as they also preserve a kind of multipotency. Thus, long after their fate as T cells is irreversibly set, mature "naïve" T cells remain competent to make a succession of additional cell type specialization decisions within the spectrum of effector T-cell subtypes that are as heritable—and often as irreversible—as true lineage choices. Furthermore, their developmental path from stem and progenitor cells is protracted compared with other hematopoietic lineages and requires a specialized microenvironment; namely, the thymus.

Major features of T-cell development appear to be conserved among all jawed vertebrates (Litman et al. 2010), albeit with some variations, and it has become clear in the last decade that a version of T-cell development also occurs in the agnathan lamprey (Bajoghli et al. 2011). The thymus itself is more phylogenetically conserved than the tissues that provide microenvironments for blood development generally (Zapata and Amemiya 2000; Boehm and Bleul 2007). Although different vertebrate classes and vertebrates at different stages of ontogeny situate the microenvironments for their main blood cell production in different anatomical sites (e.g., bone marrow, fetal liver, kidney, and gut-associated mesenchyme), they all have in common the thymus (Bajoghli et al. 2009), an epithelial organ in the neck region that is derived from branchial pouch endoderm with some neural crest contribution (Holländer et al. 2006). Furthermore, two major classes of T cells—those most easily distinguished by their use of αβ-type or γδ-type T-cell receptors (TCRs) for antigen—are found even in elasmobranchs (Miracle et al. 2001) and possibly have equivalents in lamprey (Hirano et al. 2013). Thus, T cells and their distinctive development in the thymus are nearly as much a signature feature of the vertebrate radiation as neural crest itself.

This review covers the succession of regulatory events induced by the thymus that convert broadly multpotent hematopoietic progenitors into committed pro-T cells. It focuses primary on mouse and, to a lesser extent, human data, but aspects of the process are likely to be much more broadly used.

Background: the thymic microenvironments

The structure of the mouse thymus is established between embryonic day 12 (E12) of gestation and birth and

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Corresponding author: evroth@its.caltech.edu
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then is sustained after birth beyond sexual maturity, gradually shrinking to a small but still functional remnant. T-cell export from the thymus peaks at puberty but continues well beyond the first quarter of the mouse life span. In humans, thymus organogenesis occurs at the equivalent of an earlier fetal stage, more T-cell production takes place before birth than in mice (Haynes et al. 1989), and thymic involution extends through the longer life span. However, despite the slow changes, through the early months or years of mouse or human life, respectively, the dominant components of the thymic stroma remain fairly constant, including two major types of endoderm-derived epithelium (i.e., cortical and medullary thymic epithelium) augmented by fibroblasts, neural crest-derived perivascular cells, transitory non-T hematopoietic cells, and endothelium (Foster et al. 2008; Müller et al. 2008; Takahama et al. 2017).

Hematopoietic precursors with lymphoid developmental potential migrate to the thymus while they are still multipotent. They enter the thymic cortex, where their interactions with cortical epithelial cells drive their conversion first into committed pro-T cells, then into TCR-expressing (TCR^+) cells, and then through TCR-dependent positive selection, after which they move to the medulla for further selection and maturation (Takahama 2006; Petrie and Zúñiga-Pflücker 2007; Love and Bhandoola 2011). The stages of this process in mice are distinguished based on cell surface markers as shown in Figure 1, with similar stages defined in humans (Casero et al. 2015; Canté-Barrett et al. 2017). The stroma of the thymus remains relatively constant over long periods of time as multipotent hematopoietic precursors enter it in cohort after cohort, are stimulated to begin T-cell development, proliferate, undergo commitment, and then pass through TCR gene rearrangement, selection, and maturation.

The cortical epithelium provides the known indispensable environmental signals for T-cell specification (for review, see Takahama et al. 2017). The most important of these are Notch ligand Delta-like 4 (DLL4), the cytokine Kit ligand, and the cytokine IL-7, with supporting chemokine Cxcl12 (Zamisch et al. 2005; Calderón and Boehm 2012; Buono et al. 2016). DLL4 is critically needed to interact with the Notch1 on the surfaces of the entering hematopoietic progenitors in a repeated or sustained way over multiple days in order to push the cells to shift to a T-cell-specific gene expression program. The Notch signaling that induces the T-cell program is completely asymmetrical from the start, with all of the DLL ligand expression confined to the stroma, while Notch 1 expression is required on every T-cell progenitor. First Kit ligand and then IL-7, possibly with contributions from other cytokines as well, sustain the proliferation of the hematopoietic cells as they undergo T-lineage specification and may also contribute instructive signaling to promote the T-cell program. The cortical epithelial cells make a complex lace-like structure unlike typical epithelia, which is thickly infiltrated with developing lymphocytes. This anatomy maximizes lymphoepithelial contacts that are critical both for Notch–DLL4 signaling in specification and commitment in the early T-cell developmental stages, called DN (CD4 and CD8 marker double-negative), and for later TCR–ligand contacts in selection of cells in the DP (CD4 and CD8 marker double-positive) stage.

The role of the thymic medulla is important for establishing tolerance to a wide range of tissue antigens expressed throughout the body using highly sophisticated selection mechanisms (Kyewski and Klein 2006; Takahama 2006). In the medulla, positively selected immature T cells whose TCRs interact with self-tissue antigens with too high an avidity are killed off, removing many potentially autoreactive cells. For a subset of potentially autoreactive cells, the medulla also provides special signaling interactions that can divert them instead to a protective antiaggressive "regulatory T-cell" (T_{reg}) fate. These events are important for immune system behavior throughout life and depend on the highly specialized properties of

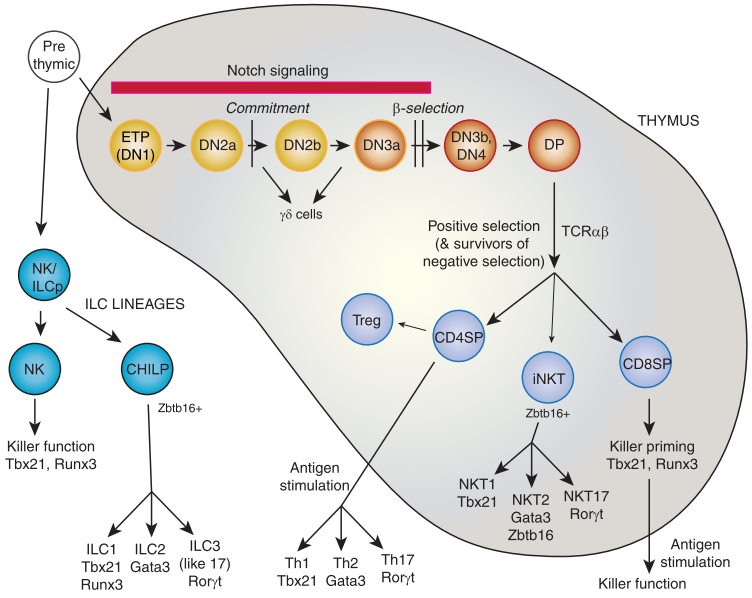

Figure 1. Schematic of T-cell development. Stages of αβ T-cell development are shown, with approximate timing of cell identity choice (gold fill), TCR gene rearrangement and selection (red–brown fill), and effector type choice (blue–gray fill; arrows). Developmental stages dependent on continued Notch signaling are indicated. For conventional αβ T cells, invariant natural killer T (iNKT) cells, and innate lymphoid cells (ILCs; lymphoid non-T cells), the distinct killer and various "helper" effector types (TH, NKT, or ILC types 1, 2, and 3 or 17) are shown with the key transcription factors that define them. T-lineage stage surface markers are as follows: (DP) $CD4^+$ $CD8^+$; (DN) $CD4^-$ $CD8^-$; (ETP) Kit^+ $CD25^-$ $CD44^+$ DN; (DN2a) Kit^{++} $CD25^+$ $CD44^+$ DN; (DN2b) Kit^+ $CD25^+$ $CD44^+$; (DN3a) Kit^- $CD25^+$ $CD44^-$; (DN3b) Kit^- $CD25^+$ $CD44^-$ $CD28^+$; (DN4) Kit^- $CD25^-$ $CD44^-$ $CD28^+$. TCRβ gene rearrangement occurs in DN3a stage. TCRα gene rearrangement occurs in DP stage. γδ T cells diverge from the αβ T-cell pathway at the stages indicated and undergo fast intrathymic effector specialization (effector type 1 and effector type 17; not shown) concomitant with TCR expression.

the medullary epithelium and other medullary antigen-presenting cells (Takahama et al. 2017). However, the medulla apparently does not participate in the initial programming of hematopoietic progenitors for a T-cell fate.

T-cell developmental mechanisms have been revealed with great help from various in vitro proxies for the thymic microenvironment. These systems have made it possible to manipulate specific variables in the cells or their environment within a discrete time window. In the 1980s and 1990s, major insights about the main themes of T-cell development and the roles of TCR specificity in T-cell selection came from use of fetal thymic organ cultures (Anderson and Jenkinson 1998). In these organ cultures, not only could whole fetal thymus lobes be studied as explants, but also the fetal thymic stroma could be depleted of developing lymphocytes, in some cases purified to separate epithelial and hematopoietic components and then reconstituted with defined progenitors from the thymus or fetal liver. An important advance in 2002 was the development of an open monolayer coculture system using an immortal stromal cell line, such as OP9, to present Notch ligand constitutively instead of thymic epithelium (Schmitt and Zúñiga-Pflücker 2002). OP9 cells themselves support only B and myeloid development but, when forced to express DLL family molecules stably, become a powerfully instructive microenvironment for T-cell specification and commitment of hematopoietic precursors, promoting development up through the generation of the earliest TCR^+ cells (Holmes and Zúñiga-Pflücker 2009). OP9-DLL1, OP9-DLL4, and analogs based on the TSt4 cell line made it possible to dissect gene regulatory network circuitry and roles of signaling and transcription factors in T-cell commitment in real time, often at single-cell resolution. Whereas these monolayer systems were inefficient at mimicking the later stages of intrathymic T-cell development, a very recent cell line-based organoid culture system has proven to support more complete T-cell development, albeit at the cost of less proliferation than in OP9-based systems (Seet et al. 2017; Montel-Hagen et al. 2019). The mechanisms described in this review have been revealed and reinforced through combined use of in vivo and in vitro microenvironments for T-cell development.

Top-down view of T-cell programming: modularity

T-cell identity and function depend on acquisition of three kinds of features that depend on different mechanisms of gene regulation. These are (1) genes that must be expressed constitutively to define a cell as a T cell, including cell surface molecules (e.g., CD3 complex components), signaling molecules (e.g., kinases Lck, Zap70, and Itk and various lineage-specific adaptor proteins), and the transcription factors needed to guarantee their expression; (2) clonally diverse antigen recognition heterodimer complexes, called the TCR, which are also expressed constitutively but cannot be encoded until the coding loci undergo an irreversible somatic recombination and mutagenesis mechanism; and (3) effector molecules that are not expressed constitutively but are poised to be activated on demand in the course of immune responses. In contrast to B cells, the main effector molecules of T cells are completely distinct from the antigen receptors that the cells use for triggering. Instead, they consist mostly of cytolytic (killing) molecules (perforin and granzymes), diverse sets of cytokines, and inducible cytokine receptors and cell-bound ligands to mediate cell–cell interaction. While genes conferring the first and second properties need to be expressed constitutively by mature T cells, those conferring the third property are normally silent by default; they are induced only upon acute immunological stimulation. Thus, T-cell programming for function includes programmed transition from silence to permanent gene expression, programmed somatic mutagenesis, and programmed poising of gene sets for expression that is distinct from expression itself.

Figure 1 introduces the stages that are recognized to distinguish developing murine T cells as they progress. In its most general terms, however, the T-cell developmental process can be broken into three major sections or program modules, which roughly correspond to meeting the three kinds of molecular requirements (Fig. 1, indicated by color).

The first module is the specification of T-cell identity. This intrathymic process is seemingly the simplest in immunological terms but is developmentally the most profound, involving wide-scale genomic activity changes and the conversion of the cells from multipotent hematopoietic precursors into cells irreversibly committed to a T-cell fate. This is the part of T-cell development that is the main focus of this review and encompasses the ETP (early T-cell precursor)–DN2b stages (Fig. 1, gold); i.e., the most immature of the DN stages (see the legend for Fig. 1 for markers used to define these stages). At the end of this process (DN3a stage), the way has been paved for the activation of the tightly regulated somatic mutation mechanism, RAG (recombinase-activating gene)-mediated recombination, that rearranges and alters the genomic DNA to encode the cells' diverse TCRs. RAG-mediated recombination provides the bridge to the second section of T-cell development.

The second module is the intrathymic process through which developing T cells are filtered—based on their newly expressed TCR—to permit only cells with physiologically "useful" TCR recognition specificities to survive and mature (Fig. 1, red–brown). The definition of "usefulness" is a key area of study in immunology beyond development per se (Starr et al. 2003; Takahama 2006; Josefowicz et al. 2012). Both chains of the TCR heterodimers are encoded by genes that depend on RAG-mediated somatic rearrangement for assembly. In the case of the more commonly used αβ TCRs, the genes encoding β chains recombine at DN3a stage, while the genes that encode α recombine later, at DP stage. Another, less-abundant class of T cells can emerge with γδ TCRs if the γ and δ TCR gene loci both rearrange successfully to encode a functional γδ heterodimer before the locus encoding β rearranges successfully (thus "γδ T cells" are distinguished from "αβ T cells" that use αβ TCRs). Because the TCR

gene rearrangement and mutation process creates many random junctional sequences, different pre-T cells emerge with often unpredictable recognition specificities or a total failure to generate functional receptors at all, so that selection is necessary. The first step of selection for αβ T cells, β selection, tests the integrity of a newly expressed β chain as a condition for progressing beyond DN3a stage. If β selection is successful, cells proliferate and progress to DP stage, then α is rearranged, and both positive selection and negative selection occur to test the quality and recognition specificity of the complete αβ combination. Positive selection allows a minority of DP to become CD4 or CD8 single-positive cells, and only those that survive negative selection emerge from the thymus as mature naive T cells. At steady state, this is the part of T-cell development that is occupying the most numerous subsets of cells in the thymus at any particular moment in time.

The third module is a complex sequence of effector differentiative specializations (Fig. 1, blue–gray). These molecular events prime mature naïve T cells for preferentially and heritably engaging one alternative of several potential effector gene expression programs in response to environmental signals. For the majority of T cells, important parts of this process usually take place after the cells leave the thymus. However, its timing is variable, as described below. The distinct T-cell effector programs involve alternative groups of effector genes, enabling one group to be coactivated in a stereotyped way that is mutually exclusive with respect to other groups of effector genes to assign the T cell to a particular effector subset. The mechanisms involve transcription factor networks and chromatin changes (e.g., see Wilson et al. 2005; Kaneko et al. 2007; Carpenter and Bosselut 2010; Oestreich and Weinmann 2012; Josefowicz 2013; Shih et al. 2014; Taniuchi 2016). Once chosen, these effector response programs are heritable, affecting the ways that clonal descendants of given mature T cells will work in the body for many months or years to come.

While these programming modules depend on different mechanisms, they are connected. For example, RAG-mediated TCR gene recombination is one outcome of the first module and the basis of the second. Similarly, for most developing αβ T cells, the effector lineage choice (module 3) takes place in two steps. First, cells undergo a choice of killer or nonkiller assignment, which occurs in close association with the TCR heterodimer-positive selection (module 2). Although the cells do not kill or mediate immune responses while still in the thymus, their future assignments can be recognized by surface marker changes, with the future killers becoming "CD8 cells" and the nonkillers becoming "CD4 cells." In the CD4/CD8 decision, the switch-like choice is mediated by a bistable transcription factor network (based on mutual repression) that is set into play by an intricate signaling process during positive selection and then maintained permanently (for reviews, see Carpenter and Bosselut 2010; Taniuchi 2016). This determines effector fate for the CD8 cells, while the full effector differentiation specialization is postponed for CD4 cells until later events. Interestingly, TCR specificity-based positive selection is also linked with effector fate specialization for γδ T cells (Prinz et al. 2013; Shibata et al. 2014; Wencker et al. 2014). However, for those γδ T cells, the kind of fate choice that they undergo in the thymus is not between killer and nonkiller fate but more closely resembles the effector differentiation of peripheral mature CD4 αβ T cells after they leave the thymus. There are yet additional minority αβ lineages generated in the thymus that also seem to undergo a γδ-like effector choice in response to positive selection, called invariant natural killer(-like) T (iNKT) cells (Engel et al. 2016; Verykokakis and Kee 2018). Thus, the relationship between module 2 selection events and module 3 functional specialization events in the T-cell development program is highly variable among cells that emerge as distinct sublineages of T cells.

Effector programming of T-cell development (i.e., the choices that cells make and their degrees of reversibility upon successive rounds of stimulation) is of extreme interest for clinical applications of T-cell biology. Modulations of these choices in mature peripheral T cells can in principle enhance the efficiency of vaccination or cancer immunotherapy or reduce the ravages of autoimmunity. Intriguingly, though, this third section of T-cell development also links mature T cells back to other classes of non-T lymphocytes at the level of gene network programming. For conventional CD4T cells in mice and humans, effector program selection is a late event that occurs only after the cells have left the thymus and long after their TCR recognition specificity has been established and quality controlled by selection; it also embodies some residual plasticity (O'Shea and Paul 2010; Shih et al. 2014). However, as already pointed out for γδ T cells and iNKT cells, the effector specialization choices that T cells make do not always wait until the mature cells leave the thymus. Most notably, in the past decade, it has become clear that cells can make these effector choices even earlier—in a very similar way but with different triggers—in cells that have never gone to the thymus in the first place and do not express any TCR complexes. The cells that share effector programs with T cells but do not express TCR are called innate lymphoid cells (ILCs) and NK cells, and the wide range of their activities in the body has become apparent only in the last decade (Diefenbach et al. 2014; Hazenberg and Spits 2014; Cortez et al. 2015; De Obaldia and Bhandoola 2015; Serafini et al. 2015). The radical variability of the timing of effector specialization relative to other steps of T-cell development is a strong indication that the effector function subprograms constitute a separate module of T-cell identity: one that relates T-cell identity to ILC identity (De Obaldia and Bhandoola 2015; Cherrier et al. 2018) and one that can be evolutionarily selected independently of the rest of the T-cell developmental sequence.

Inferring the input state: the position of T cells among hematopoietic lineages

As lymphocytes, T cells share morphological similarities with other lymphocytes such as B lymphocytes, NK cells, and nonkiller ILCs. All of these are small, round,

potentially long-lived proliferation-competent blood cells seemingly very distinct from the other immune-defensive blood cells, which are termed "myeloid" here (i.e., monocytes, macrophages, neutrophils, other granulocytes, mast cells, and dendritic cells), and at a distant extreme from the erythrocytes and platelets that exert their functions after losing their nuclei. Comparing lymphocyte cell types with each other, T cells occupy a distinctive position defined by the intersection of properties that the other lymphocytes do not share (Fig. 2A). T cells share with B cells the use of the RAG-mediated recombination and selection mechanism for somatically mutating genes that will encode their antigen recognition receptors. T and B cells even use the same RAG1/RAG2 enzymes recognizing the same short motifs in the DNA to catalyze the recombinations, although the gene complexes recombined are immunoglobulin genes in the case of B cells and TCR genes in T cells. On the other hand, as already noted, the effector functions of T cells are extensively shared not with B cells but with the NK cells and ILCs, which do not use the RAG-mediated recombination mechanisms at all.

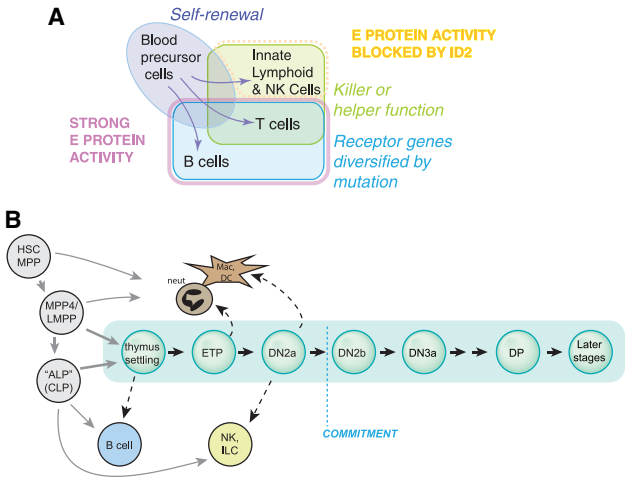

Figure 2. Relationships of the T-cell program to other hematopoietic fates. (*A*) Venn diagram showing that T cells share a common mechanism for receptor gene diversification with B cells and share a common set of killer and helper functions with NK and ILCs. The association of these character suites with E-protein activity is shown. (*B*) Persistence of access to alternative developmental pathways in T-cell precursors after entry into the thymus (light-cyan shape). Broken arrows indicate the last developmental stages at which isolated T-cell precursors can still give rise to the indicated alternative fates, provided that they are removed from the thymic microenvironment. Note that access to the B-cell option is lost two stages before access to NK and dendritic cell options. (Mac) Macrophage; (DC) dendritic cell; (Neut) neutrophilic granulocyte; (CLP) common lymphoid progenitor ("ALP" indicates a CLP that is not B-lineage-biased [Inlay et al. 2009]); (LMPP) lymphoid-primed multipotent progenitor maintaining myeloid as well as lymphoid potential (essentially similar to "MPP4" [Adolfsson et al. 2005; Wilson et al. 2008; Pietras et al. 2015]); (MPP) multipotent precursor; (HSC) hematopoietic stem cell.

The ILCs and T cells, but not B cells, include killer cell sublineages (NK and cytolytic T cells, respectively) and three corresponding sublineages of cytokine-producing "helper" cells (ILC1 and Th1, ILC2 and Th2, and ILC3 and Th17) (see Fig. 1). There are only a few CD4T cell specialties that are not yet recognized among ILCs, mostly T_{reg} cells, which suppress other cells' immune responses, and T follicular helpers (T_{FH} cells), which enter lymph node follicles to promote B-cell responses (Shih et al. 2014). The best-understood menu of shared T/ILC effector specialties is governed by shared transcription factor networks driven by Tbx21 and Runx3 for the killers and type 1 effectors, high GATA3 for the type 2 effectors, and RORγt for the type 3 effectors, respectively (Fig. 1). Supporting the idea that these are truly shared control circuits, the genome-wide chromatin accessibility states of mature mouse ILCs resemble those of stimulated mature αβ T cells of corresponding effector types (Shih et al. 2016), and, in humans, there is particularly strong agreement for circuitry and sites involving the effector polarization factors themselves (Koues et al. 2016). In contrast, the RAG recombination and selection mechanisms are highly dependent on basic helix–loop–helix E-protein transcription factors, which are active in T and B cells but neutralized in ILCs (Ikawa et al. 2006; Miyazaki et al. 2017). Thus, T cells share different detailed suites of properties with different relatives that do not share these properties with each other (Fig. 2A).

The nonhierarchical relationship between recombination/selection functions that T and B cells share and the effector functions that T cells and ILCs share knits these lymphocyte types together into a cluster relative to other hematopoietic cells. However, lymphocyte precursors have strong affinities with "myeloid" lineage cells as well (Fig. 2B). Mast cells can express many of the same effector cytokines as CD4T cells (Monticelli et al. 2005) but are thought normally to emerge from a separate myeloid-restricted precursor (Drissen et al. 2016). Remarkably, however, overexpression of the essential T-cell transcription factor GATA3 itself in precommitment T-lineage precursors can direct them to become mast cells instead within a few days (Taghon et al. 2007). Also, while post-commitment T-cell precursors and mature T cells are functionally very distinct from macrophages and neutrophils, precommitment T-cell precursors (ETP and DN2a) also naturally express substantial levels of the myeloid transcription factor PU.1, which is not only sustained through multiple cell divisions but also plays a necessary positive role in their early development (Dakic et al. 2005; Champhekar et al. 2015; for review, see Rothenberg et al. 2019). PU.1 expression is turned off during lineage commitment, as discussed below, but, before this, PU.1-expressing T-cell precursors can efficiently differentiate into granulocytes, macrophages, and dendritic cells instead of T cells if they are removed from the Notch ligands of the thymus and placed in a myeloid-supportive environment (Balciunaite et al. 2005; Bell and Bhandoola 2008; Wada et al. 2008; Yui et al. 2010; Kueh et al. 2016). Furthermore, even after commitment, experimental elevation of PU.1 can cause T-cell precursors to redirect to macrophage

or dendritic cell fates (Lefebvre et al. 2005; Franco et al. 2006; Laiosa et al. 2006). Thus, PU.1 and even GATA3, factors naturally expressed and important in early T-cell development, can also act as dose-dependent intrinsic bridges to particular myeloid fates during the early precommitment stages.

Understanding commitment and lineage restriction mechanistically depends on making rigorous distinctions between intrinsic regulatory states, environmental conditions, and expression of receptors controlling homing to different environments, all of which can influence developmental fates, as discussed in depth elsewhere (Rothenberg 2011). It is often assumed that all lymphoid cell types are obligatorily generated from a common lymphoid progenitor (CLP) that has lost all myeloid potential before making decisions among different lymphoid developmental pathways. This may be true for B-cell precursors (Zandi et al. 2012). However, in assays of intrinsic potential down to the single-cell level, intrathymic T-cell precursors have repeatedly been shown to preserve their potential to generate "myeloid" cells longer than they preserve potential to generate B cells (Bell and Bhandoola 2008; Wada et al. 2008; for review, see Rothenberg 2011). While not ruling out cells with CLP properties as one of the sources of precursors for T-cell development, this highly consistent result shows that cells do not need to lose myeloid potential before entering the thymus. When do T-cell precursors branch off from ILC precursors? ILCs are thought to be derived from a subset of CLP-like cells (Constantinides et al. 2014; Klose et al. 2014; Seehus et al. 2015; Yang et al. 2015; Seillet et al. 2016; Harly et al. 2018), but the timing of separation of the ILC precursors themselves from T and B as opposed to myeloid-competent precursors is again less clear. It may indeed be variable, with some ILCs emerging in the fetal liver or bone marrow and others branching off from T-cell precursors within the thymus (Wong et al. 2012). Thus, the T-cell program is embedded closely into a broader matrix of lymphomyeloid lineages. What are the regulatory mechanisms that not only confer T-cell properties but also separate developing T cells from these multiple alternative regulatory programs?

Transcription factors critical for establishing T-cell identity

Overview

T-cell development depends on the coordinated regulation of both broadly expressed hematopoietic transcription factors and factors preferentially expressed within the T-cell lineage. First, broadly used hematopoietic factors include Ikaros (*Ikzf1*) and Ikzf family members, Myb, Gfi1, the E proteins E2A (*Tcf3*) and HEB (*Tcf12*), and the Runx family factors. While these are modulated to higher or lower levels of expression in T-cell precursors, most of them are used at least throughout all of the stages leading to TCR expression. Second, another set of "legacy" hematopoietic progenitor factors is expressed in the precommitment (ETP and DN2a) stages of T-cell development but then down-regulated around the time of commitment. These factors include PU.1 as well as the complex of Lmo2 and Lyl1, factors that are normally silent in later T-cell development and function but appear to have a positive earlier role for T-cell precursors (for review, see Yui and Rothenberg 2014). Third, control of the activation and progression of the T-cell developmental process from one distinct step to the next depends on the sequential onset of expression of T-lineage-biased regulatory factors. In order of activation, these are first TCF1 (encoded by *Tcf7*) and GATA3 and then Bcl11b, Ets1, and the TCF1 paralog LEF1 (Supplemental Fig. S1; data not shown). These factors work together with the broadly expressed ones to drive the mechanisms that generate all types of T cells. The gene regulatory network through which these factors drive early T-cell development is complex, but its structure has begun to emerge based on current data from acute gene-specific perturbation experiments (Longabaugh et al. 2017). The modes of action of these transcription factors are discussed in detail in the following sections.

The transcriptional regulatory impacts of these specification-associated factors begin at their first expression and continue throughout their periods of activity, often shifting to regulate different T-cell genes at different phases. In the early DN stages when they can collaborate with Notch-activated RBPJ, for example, TCF1, GATA3, E proteins, and Bcl11b positively regulate viability and cell cycle control genes, genes encoding cytokine receptors, genes encoding the signaling components of the TCR complex, and the *Rag1* and *Rag2* genes encoding the recombinase itself (Supplemental Fig. S1). Peak expression of this whole ensemble of factors is achieved during the DN3 stage, shortly after commitment, as the legacy factors are repressed and TCRβ, TCRγ, and TCRδ gene rearrangement activity reaches its peak. This can be seen as the point when core T-cell identity has been determined.

The choices in later stages of T-cell development depend on a different set of factors, many of which have no apparent role in initial specification. For the majority of αβ T cells, the progression to the DP stage in which TCRα rearrangement and much TCR-dependent selection occur depends on expression of another T-cell-restricted factor, RORγt (encoded by *Rorc*), which is activated only during β selection. Later, in cells that then undergo successful positive selection of the αβ TCR, the binary choice between CD4 and CD8 fate is mediated by competition between GATA3 and ThPOK on the one hand, promoting CD4 differentiation, in mutual antagonism with Runx3 on the other hand, promoting CD8 differentiation. GATA3 is unusual in playing a powerful instructive role in functional specialization choices as well as in early T-lineage specification. For further effector differentiation of CD4, γδ, and iNKT cells, a different choice of regulatory gene sets is activated in a strongly conserved network, leading to mutually exclusive attractor states: Tbx21 for killer and type 1 effector functions, yet higher levels of GATA3 for type 2 effector functions, or reactivated RORγt for type 3 effector functions. Finally, for CD4 αβ T cells, yet another type of effector specialization can be

conferred by Foxp3 to enable the cell to exercise antiactivating T_{reg} functions in immune responses. Factors used for initial T-lineage specification, such as TCF1, E proteins, and Bcl11b, continue to play modulating or permissive roles in these later events even when they do not direct the choices themselves (Jones-Mason et al. 2012; Avram and Califano 2014; Steinke et al. 2014; Barra et al. 2015; Miyazaki et al. 2015; Kojo et al. 2017).

Notch: initiation and iterative guidance of T-cell specification

Notch signaling is essential not only to trigger the start of T-cell specification but also to prevent cells from being diverted from a T-cell fate until they traverse commitment. Then, upon commitment, Notch signaling becomes important to keep developing DN3 pro-T cells alive until β selection or γδ selection (Fig. 1, red bar). Thus, the dominant signal-dependent transcription factor in the early stages of T-cell development is the Notch1 coactivator in complex with the transcription factor RBPJ [also known as CSL or Su(H)]. Contact with microenvironmental DLL4 causes the Notch1 intracellular domain to be cleaved free from the extracellular domain and membrane anchor so that it translocates to the nucleus, where it binds with RBPJ to activate transcription. Classic Notch-activated target genes such as *Hes1*, encoding a basic helix–loop–helix repressor, are expressed throughout the specification process, although they are not T-lineage-specific. Interestingly, other Notch-dependent targets are also activated in different patterns from ETP to DN3a stage, showing that Notch signaling participates in a variety of stage-dependent regulatory ensembles (for review, see Rothenberg et al. 2016b). Only after β selection does the Notch input stop. Key regulatory genes *Tcf7*, *Bcl11b*, *Lef1*, and probably also *Gata3* are directly positively regulated by the Notch signaling pathway, although they depend on other inputs as well (see below).

TCF1 and GATA3

TCF1 (encoded by *Tcf7*) and GATA3 are indispensable for T-cell development from the first recognizable intrathymic stages. Knocking out these genes from a progenitor stage results in severe losses in population size even in ETP stage (Hattori et al. 1996; Ting et al. 1996; Hosoya et al. 2009; Germar et al. 2011; Weber et al. 2011; Scripture-Adams et al. 2014). This acute role in viability complicates identification of direct target genes, but TCF1 positively regulates *Gata3*, *Bcl11b*, the DN2 stage marker gene *Il2ra* (CD25), and genes encoding vital TCR complex and signaling components in early DN cells (Weber et al. 2011). Later, in DP stage cells, TCF1 plays a key role to collaborate with and stabilize E proteins (Emmanuel et al. 2018) and participates in many effector specialization choices (Steinke et al. 2014). Although TCF1 used in T-cell development is the same factor that can mediate Wnt signaling in other developmental contexts, in early T-cell specification, it does not appear to be transducing Wnt signals, since most evidence indicates that TCF1, but not β-catenin or γ-catenin (plakoglobin), is needed in the developing lymphocytes themselves, and deletion of the β-catenin interaction domain of TCF1 does not prevent it from supporting developmental progression (Jeannet et al. 2008; Weber et al. 2011; Xu et al. 2017). Unlike many required T-cell factors, TCF1 acts like an instructive factor for T-cell identity even in gain-of-function experiments. Artificial high-level expression of TCF1 from an early stage can accelerate progression of aspects of T-cell developmental gene expression, even activating multiple T-cell genes in prethymic precursors without concomitant Notch signaling (Weber et al. 2011) including *Gata3* itself. This presumably reflects the power of TCF1 to locate and open T-lineage regulatory sites genome-wide, as recent evidence shows that TCF1 can cause systemic chromatin changes to open a T-lineage-associated pattern of sites even in fibroblasts (Johnson et al. 2018). For reasons that are not clear, the effect of *Tcf7* disruption is milder in fetal and early postnatal waves of T-cell development than it is later, primarily affecting β selection in the earlier waves (Schilham et al. 1998). In postweaning adults and in T-cell development from adult bone marrow precursors, however, precursor numbers from the earliest intrathymic stages are affected catastrophically by loss of TCF1 (Germar et al. 2011; Weber et al. 2011).

GATA3, like TCF1, is needed for T-lineage viability from the earliest stage. *Gata3* knockouts eliminate T-cell development in fetal as well as adult mice (Hattori et al. 1996; Ting et al. 1996; Hozumi et al. 2008; Hosoya et al. 2009; Scripture-Adams et al. 2014) even though overexpression of GATA3, in contrast to TCF1, is not tolerated by mouse pro-T cells (Taghon et al. 2007; Xu et al. 2013). The growth-supporting activity of GATA3 is seen only in gain-of-function experiments under certain developmentally graded conditions, when it may also promote T lymphoma (Nawijn et al. 2001). Interestingly, human pro-T cells appear to make a more positive response to overexpression of GATA3 (Van de Walle et al. 2016), possibly connected with species differences in the response to different levels of Notch signaling (Van de Walle et al. 2013). In addition to its positive roles to make T-cell development possible, GATA3 plays a direct or indirect repressive role in an early aspect of commitment, blocking intrinsic access to the B-cell fate soon after progenitors enter the thymus (García-Ojeda et al. 2013; Scripture-Adams et al. 2014).

Bcl11b and factors activated by commitment

Despite the power and importance of GATA3 and TCF1, their expression in response to Notch signaling is not sufficient to cause T-cell lineage commitment, at least not in the mouse system. Notch signaling, GATA3, and strong TCF1 expression characterize early T-cell precursors through multiple cell divisions from the ETP through DN2a stages, yet even vigorously proliferating DN2a cells are not committed to the T-cell lineage (Yui et al. 2010; Kueh et al. 2016). Commitment does not occur until Bcl11b is up-regulated and PU.1 begins to be down-regulated, at the end of the DN2a stage. Bcl11b was discovered

as a factor required for T-cell commitment by three groups in parallel (Ikawa et al. 2010; Li et al. 2010a,b), and the timing of its up-regulation indeed coincides with commitment in individual cells, as discussed further below (Kueh et al. 2016). Although it also has key roles in nonhematopoietic contexts such as the brain, its hematopoietic expression is confined to postcommitment T-lineage cells and one set of ILCs; namely, ILC2 cells (Simon et al. 2012; Avram and Califano 2014; Califano et al. 2015; Walker et al. 2015; Yu et al. 2015).

In vivo, Bcl11b is required for survival through β selection of the developing αβ T cells, with weaker requirements in γδ cells (Wakabayashi et al. 2003; Kastner et al. 2010). However, it is not strictly required for viability in the way that TCF1 and GATA3 are; instead, it regulates appropriate thresholds for activation and lineage fidelity. If *Bcl11b* is deleted in prethymic precursors that are then allowed to differentiate in T-cell-inducing conditions with unlimited access to Notch ligands and cytokine signals, then the mutant cells can establish a very robust self-renewing population that appears to be on the brink of T-cell lineage commitment but unable to complete it (Li et al. 2010a). Data from the human system currently emphasize Bcl11b activity in the positive regulation of T-cell genes (Ha et al. 2017). In the mouse system, however, Bcl11b's repressive activity, especially against NK and ILC-associated genes, is very prominent (Longabaugh et al. 2017; Hosokawa et al. 2018a). Bcl11b-deficient pro-T cells can preserve myeloid potential (Ikawa et al. 2010; Li et al. 2010a) and inappropriately express some genes associated with stem/progenitor, myeloid, or even B cells. However, the most prominent gene expression response shifts markedly toward a partial NK or type 1 ILC gene expression phenotype (Li et al. 2010b; Longabaugh et al. 2017). If *Bcl11b* is disrupted in later T-cell precursors at the DP stage, acceleration and wholesale scrambling of gene expression associated with the CD4/CD8 lineage choice occur (Kastner et al. 2010; Kojo et al. 2017), together with loss of viability (Albu et al. 2007). Most interestingly, even *Bcl11b*-deficient pro-T cells, although blocked at a DN2–DN3-like stage, activate genes associated with mature T-cell effector specialization precociously (Longabaugh et al. 2017). This could be interpreted as the loss of a proper differentiation module timing function or as *trans*-differentiation to an innate lymphoid fate, as discussed further below. Thus, Bcl11b is vital not only for forward progression in the T-cell pathway but also for specific aspects of commitment.

The other transcription factor-coding genes up-regulated in close association with *Bcl11b*—namely, *Lef1* and *Ets1*—seem to have different effects. Deletion of *Lef1* by itself has little effect on development per se because of the overlapping stronger expression of its paralog, *Tcf7*, encoding TCF1 (a third paralog, *Tcf7l2*, is also detectably expressed before commitment). Double deletion of *Lef1* and *Tcf7* intensifies the phenotype in the fetal thymus (Okamura et al. 1998). *Ets1* disruption has a more deleterious phenotype in early T cells (Eyquem et al. 2004; Cauchy et al. 2016), but much characterization remains to be reported. Much later, the key factor that is up-regulated following β selection, RORγt, is most important at the DP stage to enable the cells to survive long enough to finish TCR gene rearrangement (TCRα gene rearrangement) and to prevent them from undergoing a premature effector immune response before they are selected (He et al. 1998; Wang et al. 2011). However, RORγt is completely silent during the T-cell specification and commitment process itself.

Runx factors: stable presence, shifting roles The expression patterns of the T-lineage-restricted factors GATA3, TCF1, Bcl11b, LEF1, and RORγt lay out a sequence of regulatory states for cells progressing from thymic immigrant through the ETP stage, to DN2 stage and through commitment, to DN3 stage and through β selection, and then to DP stage. However, throughout this process, some of the most potent regulatory effects are also mediated by broadly expressed, T-lineage-nonspecific factors that change expression very little, such as the E proteins E2A and HEB (*Tcf3* and *Tcf12*) and the Runx family factors, as shown by the severe impacts on T-cell development if these factors are deleted. Interestingly, the contributions of both Runx and E-protein factors are stage-specific even though their mRNA levels increase only a fewfold from their levels in hematopoietic multilineage progenitors. Recent data show that Runx factors and E proteins modulate and collaborate with the T-cell-specific factors through both gene network interactions and direct protein–protein interactions (Hosokawa et al. 2018a,b).

Pro-T cells initially express all three Runx family members, but Runx2 and Runx3 decline as Runx1 increases (David-Fung et al. 2009). Runx1 especially reaches its highest levels of expression recorded among hematopoietic cell types in pro-T cells around the time of lineage commitment (Heng et al. 2008; http://www.immgen.org). Knocking out CBFβ, the common binding partner of all three family members, causes catastrophic effects on entry into the T-cell lineage pathway (Talebian et al. 2007; Guo et al. 2008). Runx1 itself has a particularly notable role in commitment, not only activating the T-cell identity genes and priming the TCRβ locus for rearrangement but also becoming a repressor of *Spi1*, the gene that encodes PU.1, as described in detail below.

E proteins: the innate adaptive switch E proteins E2A and HEB are essential to prevent pro-T cells from switching to an ILC fate (Braunstein and Anderson 2011; Miyazaki et al. 2017; Wang et al. 2017). E proteins also act as direct positive regulators of numerous genes needed in T cells, including the members of the TCR signaling complex (CD3 components), kinases used in TCR signal transduction, Notch1, and the *Rag* genes themselves (Yashiro-Ohtani et al. 2009; Miyazaki et al. 2011, 2017; Del Real and Rothenberg 2013). Their role in promoting Notch1 expression itself is vital to allow ETP pro-T cells to progress to DN2 stage (Miyazaki et al. 2017). At DN3a stage, activity of E proteins not only supports T-cell gene expression but also promotes cell cycle arrest to enforce quality control at the β selection checkpoint (Engel and Murre 2004; Jones and Zhuang 2007). There are two interesting points

to note about their participation in the T-cell gene regulatory network. First, E proteins are not required to activate or sustain *Tcf7* or *Bcl11b* expression, and they even exert mild but important negative regulation of *Gata3* (Del Real and Rothenberg 2013; Xu et al. 2013; Miyazaki et al. 2017). Second, despite nearly constant expression of *Tcf3* and only gently increasing *Tcf12* from prethymic stages to β selection, the target genes of E2A and HEB are activated very steeply between DN2a and DN3a (Supplemental Fig. S1B). These genes include *Rag1* and the trio of CD3 genes: *Cd3g*, *Cd3d*, and *Cd3e*. Also, E-protein target motifs suddenly become preferentially enriched among genomic sites that open only after commitment (Johnson et al. 2018). This sharp increase in impact indicates that E-protein activity and deployment can be regulated beyond the level of transcription of E2A- and HEB-coding genes themselves.

In fact, developing T cells use numerous mechanisms to keep dynamic control of E-protein levels. When T cells are activated through the TCR, E-protein antagonists Id2 and/or Id3 are transiently up-regulated to neutralize E-protein DNA binding temporarily, and E-protein activity is restored only as the cells revert to a resting state (Bain et al. 2001). Id3 is sharply up-regulated during β selection (Yashiro-Ohtani et al. 2009). Chronically activated "innate-like" T cells such as iNKT cells maintain very high Id2 levels, comparable with those in NK cells and ILCs (http://www.immgen.org; Cohen et al. 2013). Whereas E-protein activity in normal DN2b–DN3a pro-T cells operates in a context of high Notch signaling, MAP kinase activation may enable E proteins to be degraded in response to signaling from the Notch pathway itself (Nie et al. 2003, 2008), which could also contribute to β selection. In DP thymocytes, recent evidence shows that E2A/HEB proteins not only cobind to DNA with TCF1 but are also posttranslationally stabilized by this interaction (Emmanuel et al. 2018). This stability can be important to preserve DP viability an extra day or two, long enough to generate certain rare TCRα recombinations that are important for selection to particular fates (D'Cruz et al. 2010). For effector subtype selection in the thymus and periphery, T-cell subset specializations that require different strengths of TCR signaling often require different levels of E-protein activity during the critical developmental choice points. For example, the multilevel regulation of E-protein activity contributes to the CD4/CD8 lineage choice (Jones-Mason et al. 2012) and may be essential for enabling mature T cells to distinguish between resting (naïve or memory) and activated regulatory states (Yang et al. 2011; Kaech and Cui 2012). Thus, E-protein activities must be dynamically regulated via variable antagonist expression throughout T cells' lives.

Genome-wide chromatin state shifts during T-cell commitment

T-cell commitment transforms cell potentials through a global genomic activity change. Several thousand genes change expression during this interval, up or down (Zhang et al. 2012; Mingueneau et al. 2013; Hu et al. 2018), while DNase accessibility and global genomic compartment associations change at more genomic sites than in any other stage transition between the hematopoietic stem and progenitor cells and DP thymocytes (Hu et al. 2018). This phenomenon has become increasingly clear as the timing of commitment has been refined from some time in the coarse of DN2-to-DN3 transition to the much narrower interval from DN2a to DN2b. Commitment at the single-cell level has been revealed first by distinguishing DN2 cells that did or did not express Lck-GFP transgene in a transgenic mouse line (Masuda et al. 2007) and then DN2 cells before or after a subtle reduction in expression of Kit (DN2a vs. DN2b) (Yui et al. 2010). With the advent of nondisruptive fluorescent protein alleles of the *Bcl11b* locus, it has become clear that the loss of multipotentiality coincides at the single-cell level with the onset of Bcl11b expression in late DN2a stage (Kueh et al. 2016).

Clearly, the changes in chromatin accessibility are likely to be caused by changes in transcription factor activity. The differentially enriched motifs in the sites that are open initially but close during commitment are dominated by the motif for PU.1, consistent with the expression of PU.1 in the cells until the commitment transition (Johnson et al. 2018; Ungerbäck et al. 2018). However, the most differentially enriched binding motifs in the sites that open are not those of Bcl11b, which is most sharply up-regulated at that time; instead, they are motifs for TCF1 and E proteins (Johnson et al. 2018), which are already present much earlier. Runx and ETS family motifs are about equally common in sites open before or after commitment and are highly enriched in sites bound by either PU.1 or Bcl11b (Hosokawa et al. 2018a,b; Ungerbäck et al. 2018, consistent with the complementary overlapping expression patterns of various members of these transcription factor families (David-Fung et al. 2009). What, then, alters the genomic accessibility landscape so extensively?

To answer this question, it is important to note that besides the advent of *Bcl11b*, *Ets1*, and *Lef1* expression and the graded increases in E-protein (mostly *Tcf12*) and *Runx1* expression, the majority of regulatory gene expression changes during commitment are repressive. Multiple progenitor-associated regulatory genes that are well expressed initially are silenced either concomitantly with commitment (the PU.1-coding gene *Spi1*, *Hhex*, *Bcl11a*, *Mycn*, *Mef2c*, and *Gfi1b*), before commitment (*Lmo2*), or shortly after commitment (*Lyl1* and *Erg*) (for review, see Yui and Rothenberg 2014). While expressed, these factors not only are potent developmental regulators in their own right but also collectively dominate the context within which TCF1, GATA3, and Notch signaling is first sensed by early pro-T cells and influence the binding of other factors.

Transcription factors frequently collaborate to create preferential sites for cobinding with other factors. This has been seen previously in multiple contexts; for example, (1) in a cell line resembling multilineage hematopoietic stem or progenitor cells where an ensemble of seven to 10 crucial stem cell transcription factors cobinds to regulatory sites (Wilson et al. 2010), (2) in myeloid precursors

where PU.1 and C/EBPα recruit each other to different sites (Heinz et al. 2010), and (3) in B-lineage precursors where the advent of EBF1 activity causes E2A to redistribute to sites where it can cobind with EBF (Lin et al. 2010). In a similar way, as early pro-T cells pass through commitment, GATA3 itself shifts many of its binding sites across the genome, specifically abandoning sites where it had cobound with PU.1 before commitment (Zhang et al. 2012). PU.1 may influence other factors partly due to its ability to promote opening of its target sites in chromatin, which is significant in light of its abundant binding across the genome in pro-T cells before commitment (Zhang et al. 2012; Ungerbäck et al. 2018). Open chromatin sites are likely to be easier for other factors to bind than sites in closed chromatin even though "closed chromatin" (by the criteria of transposase or DNase accessibility) need not exclude these factors absolutely from high-affinity sites (Ungerbäck et al. 2018). In addition, detailed analysis has shown that PU.1 itself also greatly influences the genomic site-binding choices of factors such as Runx1 and another factor, Satb1, through protein–protein interactions (Hosokawa et al. 2018b). Importantly, PU.1 can attract Runx1 to sites where it forms complexes with PU.1 even at the expense of vacating sites that otherwise have better Runx1 motifs. Thus, the loss of PU.1 and probably other precommitment factors not only allows the binding sites of the precommitment factors themselves to close but also frees their partners to shift to other sites, helping these new sites to open (Hosokawa et al. 2018b). These redistributional changes, caused by changes in the expression of attractive collaborating factors, provide an important way that even stably expressed factors can regulate different sets of target genes in a sharply stage-specific way.

Thus, commitment involves reciprocal changes in the expression of progenitor-associated regulatory factors, which are down-regulated, and T-lineage factors, which are up-regulated strongly, modulated upward, or stabilized into new complexes. Although some T-cell factors that appear to dominate the new genomic landscape may not change greatly in their own expression, they are brought to new sites. The result is both the loss of alternative developmental potentials and the advent of strong expression of a definitive T-cell gene expression program. The cells will undergo considerable intrathymic processing after commitment before they are set to work in peripheral immunity, but their transcriptional identities as T-lineage cells are established by the DN2b and DN3a stages immediately following commitment.

T-cell lineage commitment and TCR gene rearrangement

Commitment is not TCR-dependent; instead, it precedes most or all TCR gene rearrangement. The *Rag1* gene, encoding a key component of the recombinase complex, is a prominent member of the E-protein target gene set that is markedly up-regulated after commitment. Furthermore, both in vivo and in vitro evidence shows that commitment takes places while the cells are strongly proliferating, whereas RAG-mediated recombination depends on the cells' entering a state of G_1 arrest. The focus of the recombinases on particular target gene complexes, however, is promoted by developmentally controlled enhancer activity (for review, see Ji et al. 2010), which determines which loci will be available for recombination and can be subject to specific regulation. This enables separate regulation of the αβ and γδ TCR rearrangement programs and of the correct ordering of β rearrangement before α. Some regions of the *Tcrb* complex are evidently transcriptionally active before commitment (Chen et al. 2001; Zhang et al. 2012), and known *Tcrb* locus regulatory elements include an enhancer activated by Runx1 and Ets1 (Seo et al. 2017), but changes in TCR enhancer element activity have not been fully studied across the commitment transition. However, there is evidence that IL-7-activated Stat5 may specifically open the *Tcrg* loci (Yao et al. 2006; Maki and Ikuta 2008), and Bcl11b may have a specific role in the opening of the *Tcrb* locus because the recruitment of Runx1 to many sites throughout this locus appears to be particularly Bcl11b-dependent even before TCR gene rearrangement (Hosokawa et al. 2018a). This suggests ways that commitment-associated regulatory changes can help to guide newly available RAG proteins to the correct sites.

Lineage commitment gene network structure: *Bcl11b* as indicator

Gene network analysis should make it possible to answer two questions about commitment: (1) How does the Notch signaling in the thymus cause commitment to occur? (2) Why does the conclusion of commitment takes as long as it does? The basis for repression of the progenitor-associated legacy genes is only beginning to be understood. However, the tight linkage of *Bcl11b* up-regulation with commitment makes it possible to focus on this aspect of commitment at least by dissecting the mechanism for *Bcl11b* activation. Bcl11b is not expressed detectably by hematopoietic progenitors, CLPs, or ETP cells, and its chromatin states both on the gene body and on a distal enhancer complex are closed in these initial stages. Its activation during commitment involves a coordinated removal of repressive marks, demethylation, release from the nuclear matrix, and alterations of looping of nearly 1 Mb of DNA to bring the enhancer complex into contact with the gene body (Li et al. 2013; Isoda et al. 2017). This is a suitably discrete regulatory change to serve as a strong milestone for the commitment process, and it can be followed at the single-cell level using a *Bcl11b* fluorescent reporter mouse strain (Kueh et al. 2016).

At the time when *Bcl11b* is up-regulated, the cells have already moved to DN2a stage and proliferated as DN2a cells, usually for several cell cycles. Considerable activation of T-lineage-promoting regulators has already occurred. Notch signaling begins in ETP stage and must be sustained throughout ETP cell expansion (Tan et al. 2005) as well as through progression to the DN2a stage. Based on ETP and DN2a population phenotypes and in

vitro differentiation speeds, also, the cells activating *Bcl11b* are likely to have been expressing the genes encoding TCF1, GATA3, Runx1, E2A, and HEB (*Tcf3* and *Tcf12*) at robust levels for days (with *Runx1* and *Tcf12* expression gradually increasing). However, when they first turn on *Bcl11b*, these cells are also still expressing progenitor genes encoding the growth factor receptor Kit and the legacy factor PU.1, among multiple others (Yui et al. 2010; Yu et al. 2012; Kueh et al. 2016; Rothenberg et al. 2016a). Using in vitro T-cell differentiation on OP9-DLL1 or simple DLL1-coated plates, it has been possible to dissect not only the requirement for particular regulatory inputs but also the stage at which a given input is needed for *Bcl11b* activation (Kueh et al. 2016).

These experiments have shown that Notch signaling, TCF1, GATA3, and Runx1 are all required to enable the cells to activate *Bcl11b* (Fig. 3A; Kueh et al. 2016). The roles of these positive regulators are different, however. Notch signaling is needed to turn on *Tcf7* in the first place and may either directly activate *Gata3* or enable TCF1 to activate *Gata3*. Thus, Notch directly or indirectly induces two additional required inputs for *Bcl11b*. However, by DN2a stage, the intensity of Notch signaling affects the likelihood but not the magnitude of *Bcl11b* activation at the single-cell level (Fig. 3A, "DN2a/2b"). TCF1 and GATA3 are needed in the ETP stage to enable *Bcl11b* to be activated later; however, if they are removed during the DN2a stage before *Bcl11b* is expressed, they have little or no effect on *Bcl11b* activation. Therefore, they are needed to prime the process but not necessarily for ongoing *Bcl11b* regulation (Fig. 3A, "ETP"). Runx1 activity is the input that regulates the amplitude of *Bcl11b* expression and plays roles in both the presence and absence of Notch in pro-T cells undergoing commitment (Fig. 3A, "DN2a/2b") as well as in postthymic mature T cells. Thus, although the four positive inputs work in "AND" logic to enable the cells to turn on *Bcl11b*, their roles in the gene regulatory network and the mechanism are complementary, not equivalent (Kueh et al. 2016). Their own regulation can be seen as a feed-forward network circuit for the *Bcl11b* induction used in T-cell commitment (Fig. 3A).

The combination of requirements needed to activate *Bcl11b* in early T cells explains much of the specificity of Bcl11b expression in hematopoiesis. It does not, however, fully explain the timing, which occurs only multiple cell divisions after all of these four positive inputs are already present. One could propose several reasons why the positive inputs could be necessary and yet not sufficient, including additional positive requirements and/or repression of potential negative regulators. It has become clear recently that a strong contribution to the timing comes from a slow *cis*-acting process due to the need either (1) to relieve repressive chromatin states from the regulatory sequences for *Bcl11b* in its gene body and distal enhancer complex (Li et al. 2013), (2) to carry out a compartment flip or reposition the *Bcl11b* locus from the nuclear lamina to the nuclear interior (Isoda et al. 2017; Hu et al. 2018), or (3) both. This "epigenetic" constraint is required to explain the fact that the two different alleles of

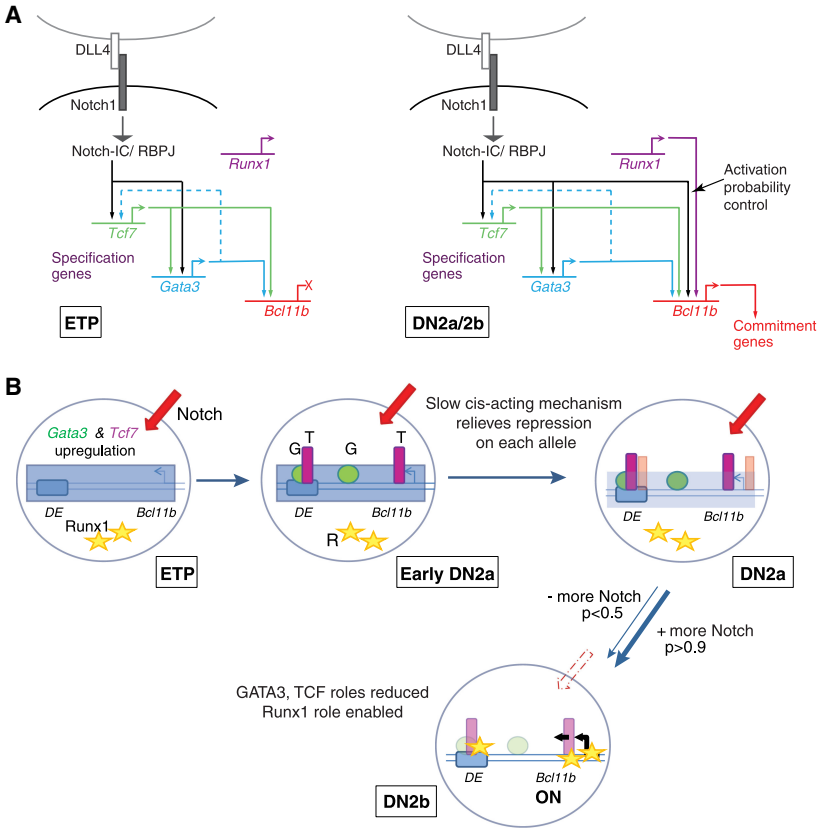

Figure 3. Molecular mechanisms controlling Bcl11b activation at the T-lineage commitment transition. (*A*) Feed-forward gene regulatory network circuitry through which Notch signaling and products of the *Tcf7* (encoding TCF1), *Gata3*, and *Runx1* genes activate *Bcl11b* through "AND" logic (Kueh et al. 2016). Activation of the Notch–*Tcf7*–*Gata3* feed-forward loop occurs in the ETP stage, but *Bcl11b* activation is delayed to DN2a/2b stage, as shown. (*B*) Model for integration of transcription factor requirements for *Bcl11b* activation with a rate-limiting chromatin opening process, which operates stochastically on each allele and imposes a delay before *Bcl11b* transcription begins for at least 2 d after *trans*-acting requirements are met (Ng et al. 2018). (Red arrow) Notch signaling; (G) GATA3 (green ovals); (T) TCF1 (product of *Tcf7*) (magenta rectangles); (R) Runx1 (yellow stars); (DE) *Bcl11b* distal enhancer; (blue rectangle) repressive chromatin state (proposed). Note that at DN2a stage, requirements of *Bcl11b* for GATA3 and TCF1 are reduced (Kueh et al. 2016), and continued Notch signaling itself is no longer essential (dashed red arrow), although it still enhances the probability of *Bcl11b* activation, as also indicated in *A*.

Bcl11b in the same cells can be activated asynchronously, with days and cell divisions often elapsing between the transitions of the two alleles in the same cells (Ng et al. 2018). This does not rule out possible involvement of yet additional *trans*-acting factors. However, fitting the known *trans*- and *cis*-acting mechanisms together into an overall model, GATA3 and TCF1 could indeed trigger priming events in ETP stage that are needed for the initiation of chromatin opening, and chromatin opening could be a slow and variable process, with later transcriptional activation mediated separately by Runx1 after the locus is open. This model is detailed in the schematic in Figure 3B.

TCF1, GATA3, and Runx1 also work together to execute another milestone event in T-cell lineage commitment; namely, the repression of *Spi1* (encoding PU.1). Despite the reciprocal changes in PU.1 and Bcl11b expression during commitment, these two factors do not appear to repress each other's transcription substantially (Del Real and Rothenberg 2013; Longabaugh et al. 2017; Hosokawa et al. 2018a; Ungerbäck et al. 2018). However, both gain- and loss-of-function experiments clearly show that GATA3 (Taghon et al. 2007; Scripture-Adams et al. 2014) and Runx1 (Hoogenkamp et al. 2007; Huang et al. 2008; Zarnegar et al. 2010; Hosokawa et al. 2018b) participate in the repression of *Spi1* around the time of commitment. Furthermore, the phenotypes of *Tcf7* knockdown cells include increased differentiation to PU.1-dependent fates, also suggesting a corepressive role for TCF1 on *Spi1* (Rosenbauer et al. 2006; Kueh et al. 2016). Thus, during commitment, GATA3, TCF1, and Runx1 work together both positively to activate *Bcl11b* and as participants in a repression mechanism needed to silence *Spi1*.

Links to other pathways and assembly of the cryptic program

Under experimental conditions in which specification proceeds but commitment fails, the changes that occur reveal other latent developmental fates that could have been supported by the initial T-cell specification process. As noted earlier (Fig. 2B), the cells that enter the thymus appear to have only transient B-cell potential, rapidly lost in the thymic environment, but significantly persisting myeloid and dendritic cell potential, NK potential, and ILC potential (Rothenberg 2011). Under normal conditions, all of these options are cut off by the time of commitment, the B-cell potential disappearing earlier in the ETP stage (Tan et al. 2005; Heinzel et al. 2007). To date, the clearest ways to perturb the commitment process have been to force PU.1 expression to continue or resume, delete or neutralize E proteins, or delete *Bcl11b*. The results show that commitment is not a unitary mechanism but rather a conjunction of distinct mechanisms that exclude different potential lineage alternatives.

Commitment can be prevented or reversed by forcing the cells to continue expressing PU.1. In this case, the NK and ILC options remain suppressed (Champhekar et al. 2015), but the cells have increased likelihoods of adopting a myeloid or dendritic cell fate, especially if Notch signaling levels drop (Franco et al. 2006; Laiosa et al. 2006; Del Real and Rothenberg 2013). Conversely, commitment can be broken by preventing the increase in E-protein activity that normally occurs at this stage. Knocking out E-protein expression or activity using either a conditional *Tcf12* and/or *Tcf3* knockout or a specifically regulated Id protein transgene has shown that loss of E protein does not restore or promote myeloid potential but rather directs developing pro-T cells to an ILC2 fate (Braunstein and Anderson 2011; Miyazaki et al. 2017; Wang et al. 2017). ILC2 cells are the ILC subset that shares with pro-T cells a requirement for TCF1, GATA3, and Bcl11b, all of which are highly expressed in DN3 cells but do not depend on E protein. ILC2 cells can normally be generated by pro-T cells up to the DN2 stage (DN2a and DN2b not separated) but not by normal DN3 pro-T cells (Wong et al. 2012); however, specific loss of E protein activity in the DN3 stage allows these cells to convert. The ILC2 identity is not simply defined by loss of E-protein target gene expression but also by additional regulatory network changes. GATA3 expression remains high, as it normally is at the DN3 stage, while there is strong upregulation of *Zbtb16*, the gene encoding PLZF (Qian et al. 2019), which is normally barely detectable in pro-T cells but is universally expressed in the precursors of all nonkiller ILCs. Thus, experimental loss of E protein unmasks the compatibility of the early T-cell specification network with early stages in ILC2 development.

Bcl11b deficiency has dramatic effects at the commitment checkpoint whether *Bcl11b* disruption occurs before or immediately after the DN2a–DN2b transition. In the mouse system, where most data are available, the most reproducible feature is the emergence of innate-like lymphoid cells, this time most often NK or ILC1-like. However, transcriptome analyses of *Bcl11b*-deficient pro-T cells show broader defects: They not only fail to activate particular sets of T-lineage genes but also aberrantly maintain stem/progenitor genes, such as *Kit*, and activate genes associated with myeloid, B-cell, and particular γδ T-cell subset programs in addition to genes involved in ILC and effector T-cell programs as described above (Longabaugh et al. 2017; Hosokawa et al. 2018a). Among the genes that Bcl11b is required to keep silent are genes encoding transcription factors used for the innate and innate-like lymphoid specification programs: *Id2*, *Zbtb16* (encoding PLZF), *Nfil3*, and *Zfp105* (an NK cell transcription factor) (Fig. 4). The fact that Bcl11b is needed to maintain silence of these genes implies that by DN2b stage, the signals and transcription factors that have been induced in the cells are also capable of mobilizing non-T (or precocious effector T) developmental programs that are kept in check only by Bcl11b. Some of these knockout effects clearly reflect specific loss of Bcl11b regulation of direct targets (Hosokawa et al. 2018a). Other effects could be exacerbated if Bcl11b plays a central role as a genome-wide chromatin-organizing factor, as some data suggest (Kojo et al. 2017; Hu et al. 2018).

Loss of Bcl11b has prominent and unexpected points of similarity with the effects of E-protein loss, positively and

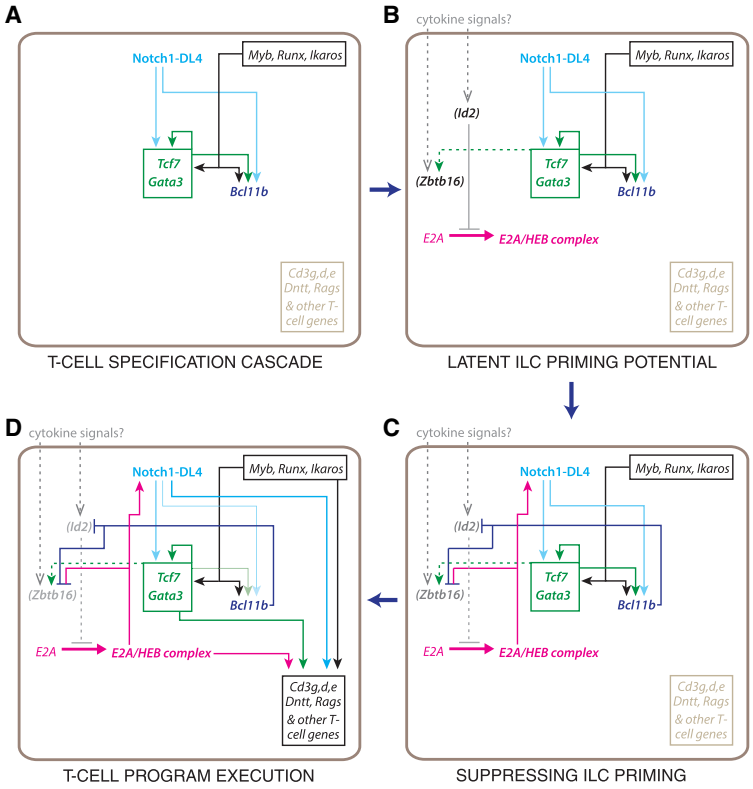

Figure 4. Bcl11b links two gene networks: a specification subnetwork based on *Tcf7* and *Gata3* and a differentiation subnetwork dependent on E proteins. The diagrams show partial gene regulatory network models highlighting events from ETP through DN2b–DN3a stages from *A* to *D* (see Longabaugh et al. 2017; Hosokawa et al. 2018a). (*A*) Notch-dependent activation events (expanded in Fig. 3A) that could be shared in T and ILC precursors. Within the Notch-activated "box," *Tcf7* (encoding TCF1) and *Gata3* are linked with mutual positive feedbacks. These events occur from ETP to DN2a stage. (*B*) The combination of TCF1 (encoded by *Tcf7*), GATA3, and probable signaling inputs from cytokines can render DN2a cells capable of activating *Id2*, *Zbtb16* (encoding PLZF), and other innate cell genes; this represents a latent ILC priming potential. If Id2 is actually expressed, it threatens to block E-protein activity needed for completing T-cell commitment. (*C*) The repression of *Id2* that Bcl11b actually exerts is T-lineage-specific and required to allow full accumulation of active E protein. The E-protein activity then has positive inputs into *Notch1*, enhancing the ability of the cells to maintain strong Notch signaling (Yashiro-Ohtani et al. 2009), and has negative inputs into *Zbtb16* and other genes in the ILC program. This panel represents events that occur in normal DN2b cells. (*D*) Execution of the full T-lineage differentiation program by collaborative regulatory actions of TCF1, GATA3, Runx1, other factors, and fully active E proteins, as seen in late DN2b and DN3a stages. Solid arrows show documented regulatory effects, broken arrows show proposed regulatory inputs for observed gene expression behavior, and grayed-out zones show connections and genes that are not active at the stages shown.

negatively (Xu et al. 2013; Longabaugh et al. 2017; Miyazaki et al. 2017; Hosokawa et al. 2018a). For example, in *Bcl11b* mutant pro-T cells, the *Cd3d*, *Cd3g*, and *Cd3e* genes are poorly activated, while genes repressed by E protein such as *Zbtb16* are activated inappropriately (Hosokawa et al. 2018a). The Bcl11b protein does not appear to bind in proximity to E2A sites in pro-T cells, but instead the convergent functions can be mediated through its gene network effects. *Id2* is a direct repression target of Bcl11b in T-lineage cells, but when Bcl11b is not present, pro-T cells in a context of unlimited Notch and cytokine stimulation are primed to activate *Id2* highly. This blocks E-protein activity, inhibiting E-protein-positive target gene expression and also allowing activation of the E-protein repression target *Zbtb16* (Hosokawa et al. 2018a). As noted above, the same T-cell specification factors that are normally needed to turn on *Bcl11b*—TCF1 and GATA3 (Kueh et al. 2016)—are vital for specification of ILC lineages as well (Yagi et al. 2014; De Obaldia and Bhandoola 2015; Serafini et al. 2015; Yang et al. 2015; Seillet et al. 2016; Harly et al. 2018), ensuring that most or all other requirements for innate lymphoid development are accessible. Thus, latent priming for a potential ILC or NK fate may lie just beneath the surface of the early T-cell program.

This role in commitment situates Bcl11b at the junction between two regulatory network subcircuits (Fig. 4). The first, governed by Notch, TCF1, GATA3, and Runx1, creates the initial committed state, signaled by Bcl11b expression (Fig. 4A); however, the factors activated in this subcircuit could also drive ILC programs instead. The second, governed by E proteins, is most important to drive postcommitment events (Fig. 4D). Bcl11b is not needed to activate but to protect the E proteins from inhibition by Id2 (Fig. 4B,C). The network position of Bcl11b reveals that the innate lymphoid program may be cryptically poised for activation by the specification cascade in the T-cell precursors as well as the T-cell program (Fig. 4B) but is not executed under normal circumstances, at least in part due to the repressive actions of Bcl11b (Fig. 4C). Bcl11b has many other repression targets besides those involved in the Id–E-protein circuit detailed here (Hosokawa et al. 2018a), and some are indeed transiently expressed at DN2a stage before Bcl11b is fully active (Longabaugh et al. 2017); their additional roles in T-cell development remain to be fully explored. However, the triple inhibitory circuit through which Bcl11b assists E proteins to repress most alternative innate programs could be vital to establish the identity of mainstream αβ T cells.

Concluding remarks

T-cell lineage choice emerges under the sustained influence of Notch signaling from a dense set of hematopoietic lineage options and yields cells that continue to make diverse refinements of their developmental roles long after their identities as T cells are fixed. Despite the

complexity of the system, its main framework and the circuitry controlling several of its most important choice points are now understood in some depth. Both the signaling events and the successive transcriptional regulation changes that guide multipotent hematopoietic progenitors into T-lineage commitment are well documented, and the perturbation approaches that are accessible in the in vitro thymic surrogate systems have made it possible to establish causality in gene network linkages. Because differentiation is linked to proliferation in this system, it has been easier to examine genome-wide site-specific impacts of transcription factor actions in later stages of T-cell commitment than at the earlier stages, which are represented by only a tiny minority of cells in the steady-state thymus. Still, this system is yielding results about the operation of dose-dependent transcriptional regulation networks and their molecular bases that can have broader application to other stem cell-based developmental systems.

There are fundamental questions that are still unanswered. First, the gene network circuitry that represses the whole set of legacy regulators during commitment probably sets the clock for differentiation timing but is only beginning to be understood. Second, although the mechanisms leading through the specification cascade to TCR gene rearrangement and selection (differentiation modules 1 and 2) are fairly explicit in terms of transcription factor interactions with chromatin, it is not clear how poising for effector function is really controlled. Once the cells are stimulated to make the choices between the four effector paths—killer, type 1 helper, type 2 helper, and type 3/type 17 helper—the key transcription factors are well known. However, it is not clear how the "same" module conferring access to these four mutually exclusive effector paths (module 3) is defined in epigenetic terms as a preferential T-lineage character long before it is used. Finally, it remains to be shown how access to this effector module is regulated differentially among lineages so that developing T cells are enabled to execute it at specific stages of development but only after module 2, depending on their subtypes, while ILCs get access to it more directly without going to the thymus, after a version of module 1 but skipping module 2 entirely. Among these effector functions are T-cell features that may have the deepest evolutionary roots (Hirano et al. 2013). Thus, the elucidation of T-cell developmental mechanisms still has more rewards to offer.

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Programming for T-lymphocyte fates: modularity and mechanisms

Ellen V. Rothenberg

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